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Zimoń, M ; Baets, J ; Auer-Grumbach, M ; et al ; Boltshauser, E

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Dominant mutations in the cation channel gene *transient receptor potential vanilloid 4* cause an unusual spectrum of neuropathies

Magdalena Zimoń,^{1,2,*} Jonathan Baets,^{1,2,3,*} Michaela Auer-Grumbach,⁴ José Berciano,⁵ Antonio Garcia,⁶ Eduardo Lopez-Laso,⁷ Luciano Merlini,^{8,9} David Hilton-Jones,¹⁰ Meriel McEntagart,¹¹ Andrew H. Crosby,¹¹ Nina Barisic,¹² Eugen Boltshauser,¹³ Christopher E. Shaw,¹⁴ Guida Landouré,^{15,16} Christy L. Ludlow,¹⁷ Rachelle Gaudet,¹⁸ Henry Houlden,^{19,20} Mary M. Reilly,²⁰ Kenneth H. Fischbeck,¹⁶ Charlotte J. Sumner,²¹ Vincent Timmerman,^{2,22} Albena Jordanova^{1,2} and Peter De Jonghe^{1,2,3}

- 1 Neurogenetics Group, VIB Department of Molecular Genetics, University of Antwerp, 2610 Antwerpen, Belgium
- 2 Neurogenetics Laboratory, Institute Born-Bunge, University of Antwerp, 2610 Antwerpen, Belgium
- 3 Division of Neurology, University Hospital Antwerp, 2650 Antwerpen, Belgium
- 4 Institute of Medical Biology and Department of Internal Medicine, Diabetes and Metabolism, Medical University Graz, 8010 Graz, Austria
- 5 Service of Neurology, University Hospital 'Marqués de Valdecilla', 'Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED)' and University of Cantabria (UC), 39008 Santander, Spain
- 6 Service of Clinical Neurophysiology, University Hospital 'Marqués de Valdecilla', 'Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED)' and University of Cantabria (UC), 39008 Santander, Spain
- 7 Paediatric Neurology Unit, Department of Paediatrics, University Hospital Reina Sofia, University of Córdoba, 14005 Córdoba, Spain
- 8 Muscle Unit, Division of Medical Genetics, Department of Experimental and Diagnostic Medicine, University of Ferrara, 44100 Ferrara, Italy
- 9 Laboratory of Musculoskeletal Cell Biology, IOR-IRCCS, 40136 Bologna, Italy
- 10 Department of Neurology, John Radcliffe Hospital, OX3 9DU Oxford, UK
- 11 Department of Medical Genetics, St. George's Hospital, Medical School, SW17 0RE London, UK
- 12 Department of Paediatrics, University of Zagreb, Medical School, University Hospital Centre Zagreb, 10000 Zagreb, Croatia
- 13 Department of Paediatric Neurology, University of Zürich, Children's Hospital, CH-8032 Zürich, Switzerland
- 14 MRC Centre for Neurodegeneration Research, Department of Clinical Neuroscience, King's College London, Institute of Psychiatry, SE5 8AF London, UK
- 15 University College London, Departments of Medicine and Neuroscience, WC1N 3BG London, UK
- 16 Neurogenetics Branch, NINDS, NIH, 3705 Bethesda, MD, USA
- 17 Laboratory of Neural Bases of Communication and Swallowing, James Madison University, 22807 Harrisonburg, VA, USA
- 18 Department of Molecular and Cellular Biology, Harvard University, 02138 Cambridge, MA, USA
- 19 Department of Molecular Neuroscience, UCL Institute of Neurology, WC1N 3BG London, UK
- 20 MRC Centre for Neuromuscular Diseases, UCL Institute of Neurology, WC1N 3BG London, UK
- 21 Department of Neurology, Johns Hopkins University, 21287 Baltimore, MD, USA
- 22 Peripheral Neuropathy Group, VIB Department of Molecular Genetics, University of Antwerp, 2610 Antwerpen, Belgium

*These authors contributed equally to this work.

Correspondence to: Prof. Peter De Jonghe,
Neurogenetics Group,
VIB Department of Molecular Genetics,
University of Antwerp, Universiteitsplein 1,
B-2610 Antwerpen, Belgium
E-mail: peter.dejonghe@molgen.vib-ua.be

Hereditary neuropathies form a heterogeneous group of disorders for which over 40 causal genes have been identified to date. Recently, dominant mutations in the *transient receptor potential vanilloid 4* gene were found to be associated with three distinct neuromuscular phenotypes: hereditary motor and sensory neuropathy 2C, scapuloperoneal spinal muscular atrophy and congenital distal spinal muscular atrophy. *Transient receptor potential vanilloid 4* encodes a cation channel previously implicated in several types of dominantly inherited bone dysplasia syndromes. We performed DNA sequencing of the coding regions of *transient receptor potential vanilloid 4* in a cohort of 145 patients with various types of hereditary neuropathy and identified five different heterozygous missense mutations in eight unrelated families. One mutation arose *de novo* in an isolated patient, and the remainder segregated in families. Two of the mutations were recurrent in unrelated families. Four mutations in *transient receptor potential vanilloid 4* targeted conserved arginine residues in the ankyrin repeat domain, which is believed to be important in protein–protein interactions. Striking phenotypic variability between and within families was observed. The majority of patients displayed a predominantly, or pure, motor neuropathy with axonal characteristics observed on electrophysiological testing. The age of onset varied widely, ranging from congenital to late adulthood onset. Various combinations of additional features were present in most patients including vocal fold paralysis, scapular weakness, contractures and hearing loss. We identified six asymptomatic mutation carriers, indicating reduced penetrance of the *transient receptor potential vanilloid 4* defects. This finding is relatively unusual in the context of hereditary neuropathies and has important implications for diagnostic testing and genetic counselling.

Keywords: *transient receptor potential vanilloid 4* gene; hereditary motor and sensory neuropathy type 2C; scapuloperoneal spinal muscular atrophy; congenital distal spinal muscular atrophy; skeletal dysplasia

Abbreviations: ARD = ankyrin repeat domain; HMN = hereditary motor neuropathy; HMSN = hereditary motor and sensory neuropathy; PCR = polymerase chain reaction; SMA = spinal muscular atrophy; *TRPV4* = transient receptor potential vanilloid 4 gene

Introduction

The long arm of human chromosome 12 harbours a region (12q21–q24) that is rich in disease-causing loci for neurological disorders (Klein *et al.*, 2003). It contains the *heat shock 22kDa protein 8* (*HSPB8*) gene linked to distal hereditary motor neuropathy type II (distal HMN II) (Irobi *et al.*, 2004), the *Ataxin 2* gene associated with spinocerebellar ataxia type 2 (Pulst *et al.*, 1996), and loci for scapuloperoneal muscular dystrophy (Wilhelmsen *et al.*, 1996) and hereditary spastic paraplegia (Schule *et al.*, 2009). In addition, loci for three other dominantly inherited peripheral neuropathies have been reported in this region, namely hereditary motor and sensory neuropathy type 2C (HMSN2C), scapuloperoneal spinal muscular atrophy (SMA) and congenital distal SMA (Isozumi *et al.*, 1996; van der Vleuten *et al.*, 1998; Klein *et al.*, 2003).

Patients with HMSN2C or Charcot–Marie–Tooth type 2C are clinically characterized by distal muscle weakness and wasting in the limbs, distal sensory loss, vocal fold paralysis and weakness of the diaphragm and intercostal muscles. The age of onset varies widely from early childhood to the third decade. Lifespan may be decreased due to respiratory complications (Dyck *et al.*, 1994; Donaghy and Kennett, 1999; Santoro *et al.*, 2002; McEntagart *et al.*, 2005). Scapuloperoneal SMA was originally described in a large New England family (DeLong and Siddique, 1992). As the name implies, patients with scapuloperoneal SMA have no detectable involvement of sensory nerves. The distribution of scapuloperoneal muscle atrophy and weakness in patients with scapuloperoneal SMA is highly unusual within the disease spectrum of hereditary neuropathies. As in HMSN2C, patients affected with scapuloperoneal SMA often have vocal fold paralysis. Congenital

distal SMA is a neurological condition with prenatal onset affecting the lower motor neurons. In a four-generation Dutch pedigree linked to the 12q23–q24 chromosomal region, the disease severity varied from congenital muscle weakness restricted to the distal lower limbs, to more severe phenotypes with weakness of pelvic girdle and trunk muscles with associated scoliosis; 15 affected members presented with arthrogryposis (Fleury and Hageman, 1985; van der Vleuten *et al.*, 1998).

Three recent studies showed that mutations in the *transient receptor potential vanilloid 4* gene (*TRPV4*) underlie these three clinically diverse hereditary neuropathies (HMSN2C, scapuloperoneal SMA and congenital distal SMA) (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landourey *et al.*, 2010). Four different mutations in eight unrelated families were reported in these studies: Arg269His, Arg269Cys, Arg315Trp and Arg316Cys. All mutations except Arg269Cys were found to be recurrent in at least two unrelated families.

Mutations were found in previously reported families including an HMSN2C family of New Zealand descent (McEntagart *et al.*, 2005) that carries the Arg315Trp mutation (Auer-Grumbach *et al.*, 2010); a HMSN2C family from the USA (Dyck *et al.*, 1994) that carries the Arg269Cys mutation as was independently shown by two studies (Deng *et al.*, 2010; Landourey *et al.*, 2010); a scapuloperoneal SMA family from the USA (DeLong and Siddique, 1992) that carries the Arg316Cys mutation (DeLong and Siddique, 1992; Deng *et al.*, 2010); and the Dutch family with congenital distal SMA (van der Vleuten *et al.*, 1998) that carries the Arg269His mutation (Auer-Grumbach *et al.*, 2010).

TRPV4 contains 15 exons and encodes a non-selective cation channel modestly permeable to Ca^{2+} (Liedtke *et al.*, 2000; Plant and Strotmann, 2007). *TRPV4* was initially identified as a

vertebrate osmosensor but can in fact be activated by a wide variety of stimuli including cell shearing, heat, endogenous agonists (e.g. endocannabinoid anandamide, arachidonic acid) and synthetic agonists like 4 α -phorbol-12,13-didecanoate (4 α PDD) (Liedtke *et al.*, 2000; Nilius *et al.*, 2003; Watanabe *et al.*, 2003; Plant and Strotmann, 2007; Everaerts *et al.*, 2009). TRPV4 shares structural features with other TRPV family members, including six putative transmembrane α -helices (TM) with a channel pore between TM5 and TM6 and intracellular domains at the N- and C- termini (Everaerts *et al.*, 2009). Based on the recently determined crystal structure of the corresponding domain from the chicken orthologue (Landourey *et al.*, 2010), TRPV4 contains an N-terminal ankyrin repeat domain (ARD) with six repeats. Such repeats have been found to be important for oligomerization, proper trafficking to the plasma membrane and interaction with other proteins (Erler *et al.*, 2004; Hellwig *et al.*, 2005; Arniges *et al.*, 2006; Everaerts *et al.*, 2009; Phelps *et al.*, 2009). A proline-rich domain is located upstream of the ARD and has been shown to be involved in mechanosensation and interaction with protein kinase C and casein kinase substrate in neurons, proteins implicated in synaptic vesicular trafficking and regulation of endocytotic processes (Cuajungco *et al.*, 2006; D'Hoedt *et al.*, 2008). A putative calmodulin-binding domain at the C-terminus is involved in Ca²⁺-dependent activation of the channel. A DAPL motif formed by four amino acids at the C-terminal end of the sequence is similar to a PDZ-binding motif (PSD95/Dlg/ZO-1), and might be responsible for interaction with proteins containing PDZ-like domains (Everaerts *et al.*, 2009).

Interestingly, TRPV4 was previously shown to be involved in human disease, as dominant mutations were reported to cause various skeletal dysplasia syndromes including brachyolmia (Rock *et al.*, 2008), spondylometaphyseal dysplasia Kozłowski type and metatropic dysplasia (Krakow *et al.*, 2009). The mutations causing these different skeletal dysplasia syndromes were shown to result in a gain of channel function (Rock *et al.*, 2008; Krakow *et al.*, 2009).

For the neuropathy-associated TRPV4 mutations there is less consensus regarding the underlying disease mechanism. Four mutations (Arg269Cys, Arg269His, Arg315Trp and Arg316Cys) were studied functionally in different cell systems (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landourey *et al.*, 2010). Two studies showed that mutant TRPV4 causes a gain of channel function with increased calcium influx compared to wild-type protein, both under basal conditions and after stimulation (Deng *et al.*, 2010; Landourey *et al.*, 2010). In contrast, the third study postulated a loss-of-function mechanism (Auer-Grumbach *et al.*, 2010).

In the current study we report one new and four previously known TRPV4 mutations. The associated phenotypic spectrum is described in eight families presenting with various forms of hereditary neuropathy. Detailed genotype–phenotype correlations giving insight into the complexity of TRPV4 neuropathies are presented.

Patients and methods

Patient cohort

To ascertain the phenotypic variability of TRPV4-related neuropathies a heterogeneous cohort was selected consisting of 145 unrelated index

patients with various types of hereditary neuropathies. For a subgroup of 83 patients, criteria for inclusion were based on the unusual clinical characteristics of the phenotypes recently described in families with TRPV4 mutations (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landourey *et al.*, 2010). Patients in this subgroup of 83 individuals were included if they were diagnosed with either hereditary motor sensory neuropathy (HMSN) or HMN with at least one of the following additional features: congenital onset (in the case of HMN diagnosis), scapular or other proximal weakness, contractures, vocal fold paralysis, diaphragmatic weakness, respiratory difficulty or pronounced scoliosis. In addition to the above-mentioned cohort of 83 patients, a second group of 62 patients with axonal forms of HMSN without specific additional features were also included.

Patients were referred from various European countries and the USA by neurologists who are well acquainted with rare neuromuscular disorders. All patients or their legal representatives signed an informed consent form prior to enrolment. The local Institutional Review Board approved this study.

Molecular genetic analysis

Genomic DNA from total blood samples obtained from patients with hereditary neuropathy and control persons was isolated using standard extraction protocols. The coding regions and exon–intron boundaries of TRPV4 were amplified by polymerase chain reaction (PCR) using primer oligonucleotides designed with Primer3 (Rozen and Skaletsky, 2000) (Supplementary Table 1, PCR conditions are available upon request). PCR products were purified with the exonuclease I-shrimp alkaline phosphatase enzyme (USB, Cleveland, USA). Mutation screening was performed by bidirectional sequencing using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Fragments were electrophoretically separated on an ABI3730xl DNA Analyser (Applied Biosystems, Foster City, USA). Sequence analysis was performed with the SeqMan[™]II (DNASTAR Inc., Madison, USA) program. The nucleotide numbering was based on the published online protein (NP_067638) and mRNA (NM_021625) sequence of TRPV4 (<http://www.ncbi.nlm.nih.gov>). For the description of the mutations we used the latest conventions of the Human Genome Variation Society nomenclature (<http://www.hgvs.org/mutnomen>). Sequence variants were confirmed by an independent PCR and sequencing of the original or new stock DNA samples. Segregation of the mutations with the disease phenotype was analysed in all families. For the Arg232Cys and Val620Ile mutations, 280 controls of European descent were screened.

Haplotype sharing and paternity testing

In order to investigate haplotype-sharing among the families carrying the Arg232Cys or Arg269Cys mutation and confirm the *de novo* character of the Val620Ile mutation, genotyping was performed using five highly informative short tandem repeat markers surrounding the TRPV4 gene (D12S105, D12S1583, D12S1645, D12S1343 and D12S1344). Short tandem repeats were PCR-amplified with fluorescently labelled primer pairs; sequences are available at <http://www.ncbi.nlm.nih.gov>. The PCR fragments were mixed with formamide and the GeneScan[™] 500 Liz[®] Size Standard (Applied Biosystems, Foster City, USA) and subsequently size-separated on an ABI3730xl DNA Analyser. Genotypes were analysed using Local Genotype Viewer, an in-house developed software program (<http://www.vibgeneticservicefacility.be/>).

Paternity was tested using 15 highly informative short tandem repeats distributed throughout the genome (ATA38A05, D1S1646,

D1S1653, D1S1360, D2S2256, D3S3037, D4S2382, D4S3240, D7S509, D8S1759, D9S1118, D12S1056, D12S2082, D16S2619 and GATA152H04). The short tandem repeat fragment analysis was processed with the same equipment and software.

Results

Genetic findings

Mutation screening in a large cohort of 145 unrelated patients with various types of hereditary neuropathies revealed eight families with five different heterozygous missense mutations in *TRPV4* (Table 1 and Fig. 1) including the American 'Family 1' (F1) (Landoure *et al.*, 2010). The other seven families were not previously reported to carry a *TRPV4* mutation. Autosomal dominant segregation of the mutations was found in seven families; the remaining index patient (PN-1394.1) was sporadic and carried a *de novo* mutation (Fig. 2). Affected members in a UK family (CMT-455), a Swiss family (CMT-456) and one patient from a UK pedigree (CMT-1100) carried a novel sequence variation in exon 4 (c.694C>T) resulting in an Arg232Cys transition in the TRPV4 protein. The Arg269Cys (c.805C>T) mutation in exon 5 was present in the previously reported American family (F1) (Landoure *et al.*, 2010) and in a new Spanish family (CMT-858). Three other previously identified mutations were found: Arg269His (exon 5) in an Italian kindred (CMT-165) and Arg315Trp (exon 6) in a third UK family (CMT-149) (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landoure *et al.*, 2010). Finally the Val620Ile (c.1958G>A) mutation in exon 12, which was previously reported to cause autosomal dominant brachyolmia (Rock *et al.*, 2008), was identified in an isolated Croatian patient (PN-1394.1). The Arg232Cys and Val620Ile mutations were absent from 280 control individuals of European descent.

Segregation analysis was performed in all families and *TRPV4* mutations were present in all available affected individuals (Fig. 2). Genetic testing revealed three asymptomatic carriers of the Arg269Cys mutation in pedigree CMT-858. These individuals (CMT-858.02, CMT-858.07 and CMT-858.08) were clinically unaffected at the age of 79, 36 and 6 years, respectively. Furthermore, two unaffected members from the F1 family (II.4 and IV.6 in the original paper) also carried the Arg269Cys mutation (Landoure *et al.*, 2010). In family CMT-455 one individual (CMT-455.04) carrying the Arg232Cys mutation was clinically unaffected at the age of 69 years.

Additional short tandem repeat marker and single nucleotide polymorphism analysis for the recurrent Arg232Cys mutation (families CMT-455, CMT-456 and CMT-1100) and Arg269Cys mutation (CMT-858 and F1) showed that the Arg232Cys mutation in families CMT-455 and CMT-1100 resides on a common haplotype. The other families do not share haplotypes (Table 2).

Four of the five mutations identified in this study target conserved residues in the ARD of TRPV4 based on the crystal structure of ARD of the TRPV4 chicken orthologue (Landoure *et al.*, 2010). The mutant residues Arg232, Arg269 and Arg315, and previously identified Arg316 (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010) are situated on the convex surface of ARD in the loops of finger 2, 3 and 4, respectively (Fig. 3A). Mapping of the sequence conservation among 27 TRPV4 orthologues on the molecular surface demonstrate that the convex face of the ARD is very highly conserved (Fig. 3B). The solvent-accessible electrostatic surface properties show that the convex surface is also highly positively charged (Fig. 3C).

Clinical findings

The first subgroup of the screening cohort consisted of 83 patients presenting with various hereditary neuropathies with at least one of the additional features as described above. For 15 patients of

Table 1 Families with *TRPV4* mutations identified in this study

Mutation	Amino acid change	Exon	Known phenotypes	Family ^a	Country of origin	Familial history	Number affected	Additional remarks
c.694C>T	Arg232Cys	4	HMN with vocal fold paralysis, SPSMA, HMSN2	CMT-455 CMT-456 CMT-1100	UK Switzerland UK	AD AD AD	3 4 2	One unaffected mutation carrier (CMT-455), new mutation. CMT-455 and CMT-1100 share the same haplotype (Table 2)
c.805C>T	Arg269Cys	5	HMSN2C, SPSMA, congenital distal SMA	CMT-858 F1	Spain USA	AD AD	2 7	
c.806G>A	Arg269His	5	HMSN2C, SPSMA, congenital distal SMA	CMT-165	Italy	AD	3	
c.943C>T	Arg315Trp	6	HMSN2C, SPSMA, congenital distal SMA	CMT-149	UK	AD	5	Three unaffected mutation carriers. Two unaffected mutations carriers.
c.1858G>A	Val620Ile	12	HMSN2, autosomal dominant brachyolmia	PN-1394	Croatia	IC	1	

AD = autosomal dominant; IC = isolated case; SPSMA = scapuloperoneal spinal muscular atrophy.

a: This study.

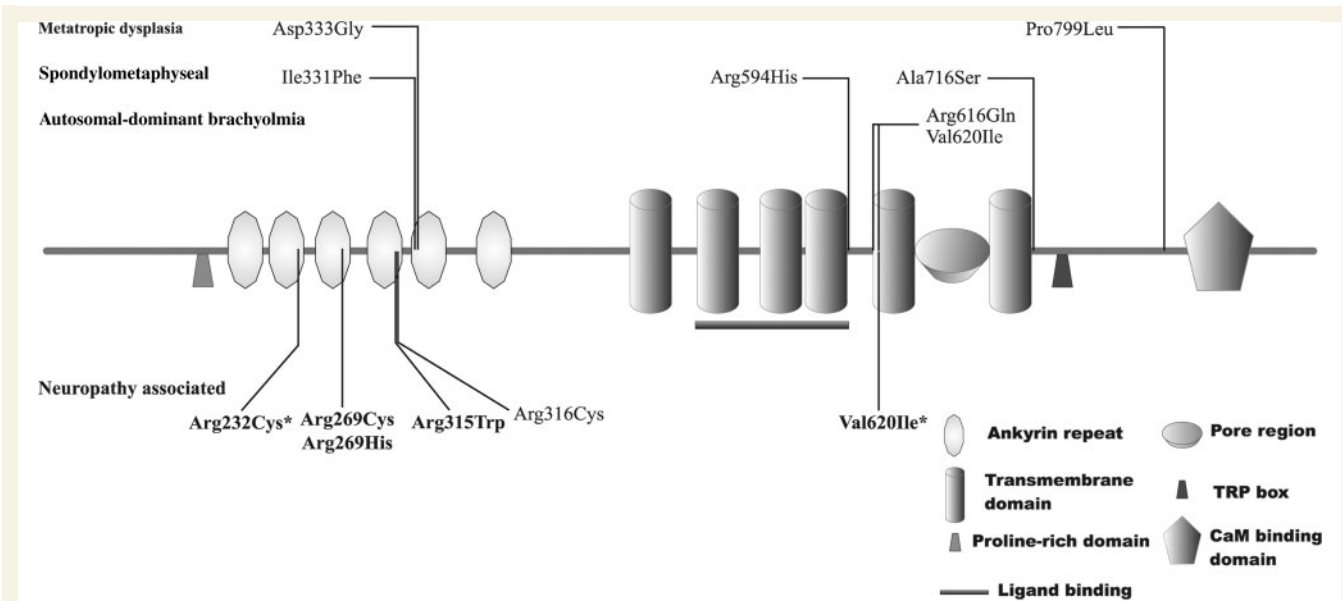


Figure 1 Location of mutations in the different domains of TRPV4 protein. Neuropathy causing mutations are indicated as well as previously described mutations for autosomal-dominant brachyolmia, spondylometaphyseal dysplasia (SMDK), Kozłowski type and metatropic dysplasia. Mutations identified in this study are in bold. The new neuropathy associated mutations are indicated by asterisks.

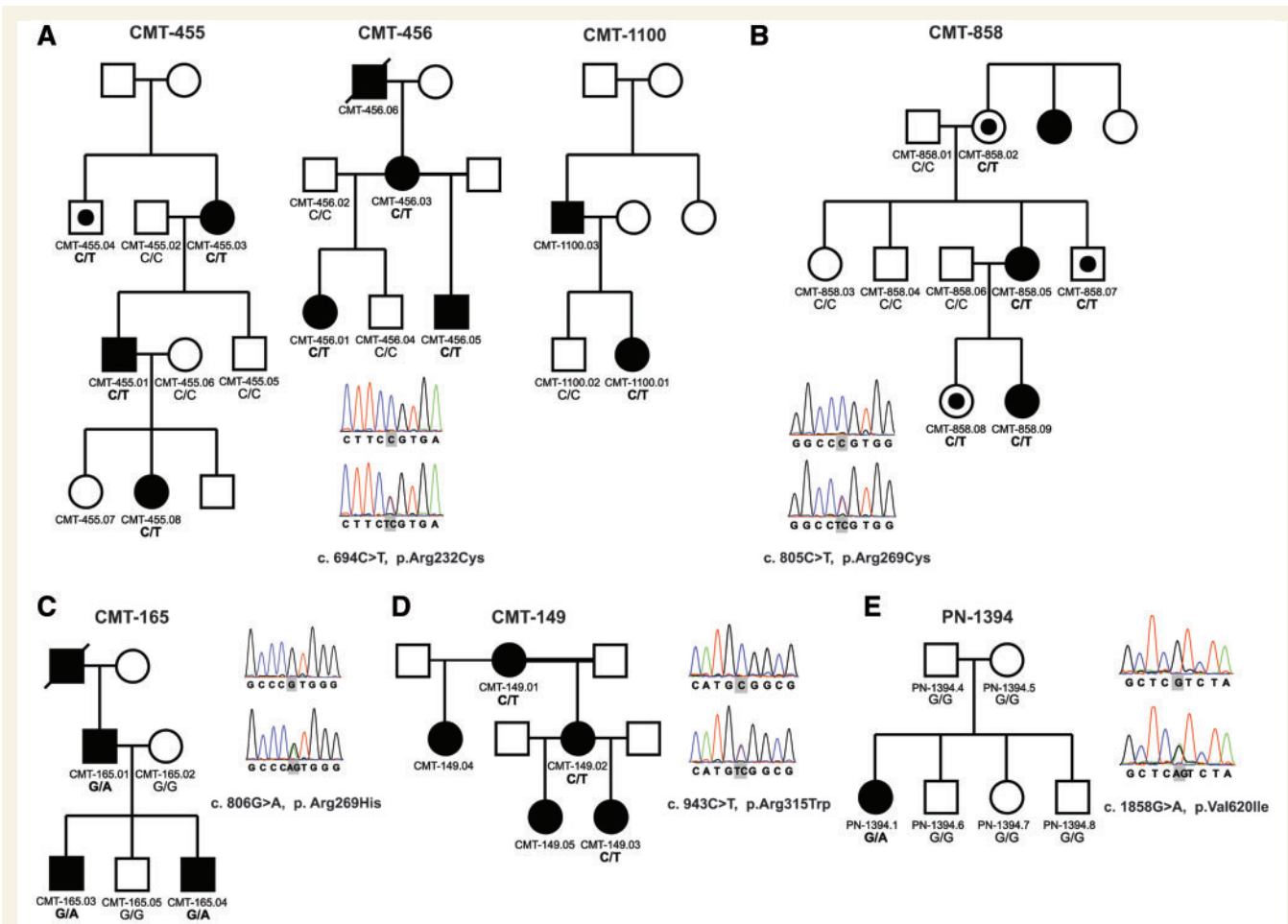
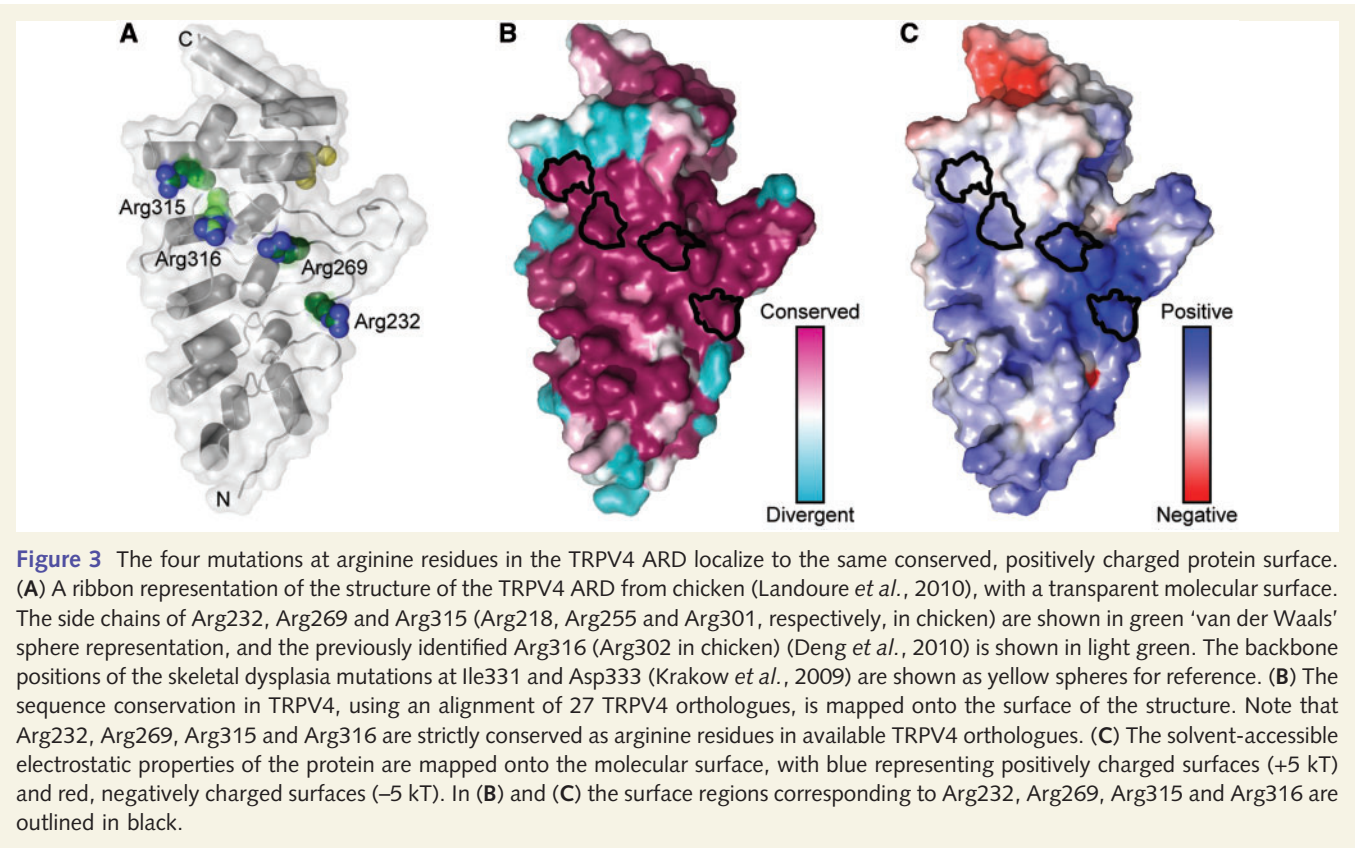


Figure 2 Pedigrees of families with TRPV4 mutations. Segregation analysis and sequence trace files is shown for seven families, the pedigree of family F1 is shown elsewhere (Landouze et al., 2010). (A) Families CMT-455, CMT-456 and CMT-1100 carrying the Arg232Cys mutation. (B) Family CMT-858 with the Arg269Cys mutation. (C) Family CMT-165 with the Arg269His mutation. (D) Family CMT-149 with the Arg315Trp mutation. (E) Family PN-1394 with the Val620Ile mutation. Square = male; circle = female; black filled symbol = affected; empty symbol = unaffected; empty symbol with black dot = unaffected mutation carrier. Genotype is indicated under each individual from whom the DNA was available for testing.

Table 2 Haplotype analysis of 12q markers in patients with Arg232Cys and Arg269Cys mutations in *TRPV4*

Patient Marker	Arg232Cys			Arg269Cys	
	CMT-455.01	CMT-456.01	CMT-1100.01	CMT-858.09	F1.IV.4
D12S105	143	147	145	145	143
D12S1583	237	225	237	221	239
D121645	212	216	212	218	212
rs3742032 (T/C)	C	T	C	C	T
rs3825394 (A/C)	C	A	C	C	A
D12S1343	206	206	206	206	208
D12S1344	239	235	239	239	227

The alleles of the short tandem repeats are sized in base pairs. The position of the markers is according to the reference assembly of NCBI genome build 36.3.



this subgroup, a formal diagnosis of HMN was made; HMSN1 for 26 and HMSN2 for 16. For the remaining 26 patients of the first group of 83, no detailed electrophysiological information was available so no clear distinction between HMN, HMSN1 and HMSN2 could be made. Disease onset ranged widely from childhood through adulthood; nine HMN patients had a congenital onset. Scapular or other proximal upper limb weakness was encountered in eight patients and contractures were present in 17 patients. In this cohort 14 patients were diagnosed with vocal fold paralysis, five with diaphragmatic paralysis and 36 with scoliosis. The second subgroup of the screening cohort consisted of 62 patients with a diagnosis of HMSN2 without additional features. The detailed clinical and electrophysiological findings in the eight families are shown in Tables 3 and 4. Data from 27 patients

were included; 23 had genetically confirmed *TRPV4* mutations, the remaining 4 were affected by history but DNA for molecular testing was not available. Clinical findings and linkage data in family CMT-456 were reported earlier (Boltshauser *et al.*, 1989; McEntagart *et al.*, 2002). Detailed laryngoscopy assessment of vocal fold function was performed in the F1 family showing that the vocal fold involvement was often asymmetric (Table 5). A video fragment from the laryngoscopic evaluation of vocal fold dysfunction in patient F1 III.2 is provided in the Supplementary material. The clinical phenotype of the patients described in the current study is highly variable both between and within families. The majority of patients seem to fall in the category of pure or predominantly motor nerve disorders. Nerve conduction studies

Table 3 Clinical characteristics of patients with proven *TRPV4* mutations

Patient	Age at onset	Age at last examination (years)	Symptoms at onset	Weakness lower limbs	Weakness upper limbs	Weakness	Vocal fold paresis	Scoliosis	Contractures	Sensory involvement	Additional features	Diagnosis
				Distal	Proximal	Distal	Proximal	Other				
CMT-455.01	14 y	33	Stridor	+	—	—	—	Left diaphragmatic paralysis	+	—	Respiratory insufficiency, pes cavus	Distal HMN with vocal fold paresis
CMT-456.01	6 m	30	Stridor	+	+	+	—	Scapular winging	+	Unilateral L	Sensorineural hearing loss, arthenoidectomy L at 10y	Scapuloperoneal SMA
CMT-456.03	Childh	52	Weakness hands	+	+	+	—	—	+	Unilateral L, asymptomatic	Sensorineural hearing loss, loss of myelinated fibres on sural nerve biopsy	Distal HMN with vocal fold paresis
CMT-456.05	Childh	36	Distal weakness upper/lower limbs	+	—	+	—	—	+	Unilateral, dysphonia	Sensorineural hearing loss	Distal HMN with vocal fold paresis
CMT-456.06	Adol	65	Weakness hands	+	—	+	—	—	+	40 y, unilateral L	Bilateral hearing aids at 40 y	Distal HMN with vocal fold paresis
CMT-1100.01	14 y	27	Scoliosis	+	(4)	—	+	—	+	Distal ↓	—	HMSN2
CMT-1100.03	11 y	43	Tripping with feet	+	+	+	(4)	Truncal	—	Distal UL/LL	Wasting shoulders and mid thigh, pes cavus, restless legs syndrome	HMSN2
CMT-858.05	Childh	39	Scapular winging	+	(2)	—	+	Scapular winging, mild facial weakness	+	Distal ↓	Pes cavus, hammetoes	Scapuloperoneal SMA
CMT-858.09	Cong	6	Arthrogryposis lower limbs	+	(2)	+	(3)	Shoulder anyotrophy	+	Arthrogryposis lower limbs	Respiratory insufficiency, tracheostomy, bladder incontinence	Congenital distal SMA
F1 III.2	44 y	71	Distal weakness lower limbs	+	(3, 4)	—	+	—	+	Minimal	Sensorineural hearing loss, bladder urgency	HMSN2C
F1 IV2	5 y	48	Distal weakness lower limbs	+	(1)	+	(4)	—	+	Mild	Pes cavus, bladder incontinence, CS	HMSN2C
F1 IV3	5 y	46	Stridor	+	(2)	+	(4)	Neck flexor (4)	+	Mild	Mild dysphagia, postural tremor, bladder urgency and incontinence, mild pes cavus, CS	HMSN2C/distal HMN
F1 IV4	3 y	44	Distal weakness lower limbs	+	(0)	+	(1)	Neck, truncal, diaphragm, tongue muscles, facial	+	(Elbow, fingers, wrists, knees, hips)	Sensorineural hearing loss, bladder incontinence, CS	HMSN2C
F1 V1	7 y	24	Distal weakness lower limbs	+	(4)	—	+	Neck flexor (4)	+	Mild	Pes cavus, hammetoes	HMSN2C
F1 V3	5 y	20	Stridor	+	(4)	—	+	Neck flexor (4)	+	Mild	Bladder urgency, pes cavus, hammetoes, CS	HMSN2C
F1 V8	2 y	18	Stridor	+	(4)	—	—	—	+	Minimal	Pes cavus	HMSN2C/HMN
CMT-165.01	Childh	52	Distal weakness lower limbs	+	+	—	(mild)	Scapular winging SCM, trapezius,	—	—	—	Scapuloperoneal SMA
CMT-165.03	Cong	27	Club feet	+	(1)	—	+	SCM, trapezius	—	—	Mild lumbar hyperlordosis, muscle hypertrophy UL plus deltoids and pectoralis, finger tremor	Congenital distal SMA
CMT-165.04	8 y	10	Distal weakness lower limbs	+	(1, 2)	—	—	SCM, trapezius, neck flexors	—	—	—	Scapuloperoneal SMA
CMT-149.01	>20 y	51	Scoliosis	+	—	—	—	Scapular winging R, neck flexors	+	—	Facial asymmetry	Scapuloperoneal SMA

(Continued)

Table 3. Continued

Patient	Age at onset	Age at last examination (years)	Symptoms at onset	Weakness			Vocal fold paresis	Scoliosis	Contractures	Sensory involvement	Additional features	Diagnosis
				Distal	Proximal	Distal						
CMT-149.02	Cong	28	Club feet	+	+	+	NA	+	(Lumbar) Talipes equinovarus	—	Facial asymmetry, tongue fasciculations, dorsal hyperlordosis	Congenital distal SMA
CMT-149.03	Cong	11	Club feet	+	(L>R)	—	—	—	Talipes equinovarus, knee L	—	—	Congenital distal SMA
CMT-149.04	Child	12	Pain lower limbs	+	—	—	—	—	—	—	—	Scapulohumeral SMA
CMT-149.05	Cong	4	Club feet, stridor	+	—	—	+	—	Talipes equinovarus	—	Gastrostomy	Congenital distal SMA
PN-1394.1	6 y	15	Distal weakness lower limbs	+	—	—	—	+	Ankles and knees	—	Short stature	HMSN2

+ = present; — = absent; y = year; m = month; childh = childhood; cong = congenital; adol = adolescence; L = left, R = right; SCM = sternocleidomastoideus muscle; NA = information not available; CS = cold sensitivity with worsening of weakness in hands; UL = upper limbs; LL = lower limbs.

showed an axonal neuropathy with preserved or only slightly attenuated nerve conduction velocities in all studied patients (Table 4).

Of special interest is the isolated Croatian patient PN-1394.1 who developed symptoms of a predominantly motor neuropathy in early childhood. Nerve conduction studies were compatible with an axonal neuropathy and concentric needle EMG showed chronic neurogenic changes in distal muscles (Tables 3 and 4). Because scoliosis and short stature in this patient might resemble a brachyolmia phenotype, additional radiographic studies were performed. A right convex kyphoscoliosis with flattened vertebrae in the mid-thoracic segment was found and the necks of both femurs were shortened, wide and slightly irregular. There was no brachydactyly or delay of the carpal bone ossification. Genetic testing for other more common causes of hereditary neuropathy (CMT1A duplication and point mutations in *PMP22*, *MPZ*, *GJB1* and *SH3TC2*) was unremarkable in this patient.

Discussion

In this study we report clinical and genetic findings in eight unrelated families with hereditary neuropathies caused by *TRPV4* mutations identified during mutation screening of a heterogeneous cohort of 145 unrelated index patients. The F1 family was already reported to carry a mutation in *TRPV4* but is described in detail here (Landourey *et al.*, 2010). In the current literature there are at least two more families that may link to the 12q-locus, however they were not yet reported to carry a *TRPV4* mutation. One is of UK origin (Donaghy and Kennett, 1999; McEntagart *et al.*, 2005); the second is of Italian descent (Santoro *et al.*, 2002). Five different heterozygous mutations were identified in the current study, of which four (Arg269Cys, Arg269His, Arg315Trp and Val620Ile) were previously reported in other families (Rock *et al.*, 2008; Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landourey *et al.*, 2010) (Table 1). The novel Arg232Cys mutation was found in three families who were unrelated by history. Two of these (CMT-455 and CMT-1100) are probably distantly related since they share the same disease haplotype. The third family with the Arg232Cys mutation does not share this haplotype. No common haplotype could be found in pedigrees CMT-858 and F1, both carrying the Arg269Cys mutation (Table 2). Thus, all mutations in this study are recurrent in at least two families pointing to the presence of mutation hotspots in exons 4, 5, 6 and 12 of *TRPV4* (Rock *et al.*, 2008; Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landourey *et al.*, 2010). In addition, the recurrent Arg594His mutation was described in spondylometaphyseal dysplasia Kozlowski type. This points to another mutational hotspot in exon 11 (Krakow *et al.*, 2009).

Reduced penetrance was observed in six asymptomatic mutation carriers, for the Arg232Cys mutation in family CMT-455 and for the Arg269Cys mutation in families CMT-858 and F1 (Landourey *et al.*, 2010) (Fig. 2). It was also reported previously for the Arg315Trp mutation (Auer-Grumbach *et al.*, 2010). Incomplete penetrance is a relatively uncommon event in the context of hereditary peripheral neuropathies, although it has been observed before for *glycyl-tRNA synthetase* (*GARS*) (Sivakumar *et al.*, 2005), *myelin protein zero* (*MPZ*) (De Jonghe *et al.*, 1999) and *Berardinelli-Seip*

Table 4 Nerve conduction studies in patients with proven *TRPV4* mutation

Patient	Age (years)	Median motor		Ulnar motor		Peroneal motor		Tibial motor		Median sensory		Ulnar sensory		Sural sensory	
		Amp 4.0	CV 49.0	Amp 4.0	CV 49.0	Amp 3.0	CV 41.0	Amp 3.0	CV 41.0	Amp 7.0	CV 46.0	Amp 2.0	CV 47.0	Amp 1.0	CV 44.0
CMT-455.01	33	—	—	7.6	55.0	—	—	4.2	49.0	9.4	N	10.3	N	11.0	N
CMT-455.08	10	—	—	—	—	—	—	0.9	39.7	—	—	—	—	12.8	N
CMT-456.01	13	7.0	50.0	—	—	—	—	12.0	45.0	10.0	55.0	—	—	—	—
CMT-456.03	35	13.0	61.0	—	—	—	52.0	10.0	49.0	10.0	52.0	10.0	49.0	8.0	54.0
CMT-456.05	33	5.3	54.0	—	—	1.6	49.0	—	—	—	—	—	—	1.6	45.0
CMT-1100.01	23	7.2	57.0	4.2	65.0	1.6	55.0	3.9	—	7.0	54.0	5.0	58.5	5.0	50.0
CMT-1100.03	42	—	60.0	—	66.0	0.4	—	—	—	A	—	—	—	A	—
CMT-858.05	40	3.8	52.9	3.6	51.4	0.9	53.7	0.5	52.3	8.4	56.9	—	—	18.1	56.3
CMT-858.09	4	4.6	49.1	1.6	52.4	0.4	48.8	0.4	44.7	9.0	47.8	—	—	6.5	48.1
F1 III2	71	4.9	57.0	7.0	51	2.1	46.0	4.5	41.0	13.0	36.0	18.0	46.0	—	—
F1 IV3	45	0.4	50.0	A	A	A	A	1.4	40.0	5.0	56.0	3.0	52.0	9.0	41.0
	27	3.4	53.6	1.8	62.5	0.3	51.6	—	—	10.0	60.0	5.0	52.3	9.0	46.1
F1 IV8	19	12.4	56.0	—	—	4.1	40.0	6.5	45.0	20.0	50.0	—	—	13.0	41.0
CMT-165.03	22	—	—	—	—	0.6	45.0	—	—	—	—	—	—	23.0	47.0
CMT-165.04	8	1.7	52.0	—	—	—	—	—	—	—	—	—	—	—	—
CMT-149.01	47	3.0	59.0	—	—	—	—	5.2	50.7	N	N	N	N	N	N
CMT-149.02	29	7.0	51.0	—	—	—	—	—	—	4.5	N	3.2	N	—	—
CMT-149.03	1	—	53.3	—	—	—	—	—	—	—	—	—	—	—	—
CMT-149.04	12	—	—	—	—	—	—	11.9	—	—	—	—	—	6.9	N
PN-1394.1	16	8.0	55.7	N	N	0.05	33.3	—	—	10.0	50.0	N	N	A	A

A = absent response; Age = age at examination; Amp = amplitude (motor: in mV; sensory: in μ V); CV = conduction velocity (in m/s); — = not measured; N = normal. Bold indicates abnormal values.

Table 5 Laryngoscopic findings in family F1

Patient	Dyspnoea	Aspiration	Dysphonia	Vocal fold adduction		Vocal fold abduction		Comments
				Left	Right	Left	Right	
F1 III2	—	+	—	1	1	1	1	Raspy voice
F1 IV3	+	+	+	2	1	2	1	High-pitched voice
F1 V8	+	—	+	2	2	2	1	Husky voice, arytenoidectomy at age 2 years

+ = present; — = absent. Vocal fold movement: 0 = normal; 1 = impaired mobility; 2 = immobile.

congenital lipodystrophy 2 (BSCL2) (Auer-Grumbach *et al.*, 2005). In *TRPV4*-associated neuropathy, however, reduced penetrance seems to be a relatively common feature that is not mutation-specific.

In accordance with previous descriptions, further phenotypic subdivision could be made: congenital distal SMA with contractures, scapuloperoneal SMA, HMSN2C (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landoure *et al.*, 2010), but also HMN with vocal fold paralysis and 'generic' HMSN2 (Table 3). Taking the previously described mutations and phenotypes into account and the fact that different phenotypes can arise even within one family, no clear correlations can be made between the various *TRPV4* mutations and the associated phenotypes (Table 1). Although the separate disease entities of *TRPV4*-associated neuropathies could still serve a purpose from a semiological point of view, they are actually elements of a neuropathy disease spectrum that is exceptionally broad.

Mutations in *TRPV4* were mainly identified in complex phenotypes. Index patients from seven out of our eight families

presented with neuropathies with additional features, as opposed to one index (family CMT-1100), who was diagnosed with HMSN2 without clear additional features. The mutation frequency can therefore be estimated at 8.4% in the subgroup of the complex neuropathies (7 out of 83) and only 1.6% in the subgroup of the HMSN2 neuropathies without additional features (1 out of 62). The unifying feature however of all identified neuropathy patients with *TRPV4* mutations is the axonal electrophysiology. This is further underscored by the fact that our screening cohort also contained 26 index patients with an electrophysiological diagnosis of demyelinating neuropathy (HMSN1), none of which was found to have a *TRPV4* mutation.

Of special importance for the prognosis of patients is the frequently observed vocal fold involvement. Nearly all families that have been reported to date with *TRPV4* mutations contained patients with vocal fold dysfunction (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landoure *et al.*, 2010). In five out of eight families in our series, vocal fold dysfunction was observed, often unilateral or asymmetric with the left more affected than the right

vocal fold (families F1, CMT-456 and CMT-858) and ranging in severity from asymptomatic in patients CMT-456.03 and CMT-858.05 to clear respiratory insufficiency requiring tracheostomy in patient CMT-858.09 (Tables 3 and 5 and Supplementary video fragment). Some patients underwent surgical procedures of the vocal folds (families F1 and CMT-456). Vocal fold involvement is not a typical feature of hereditary neuropathy in general, but it has occasionally been associated with recessive *ganglioside-induced differentiation-associated protein 1* (*GDAP1*) mutations (Sevilla *et al.*, 2003; Stojkovic *et al.*, 2004), dominant *early growth response 2* (*EGR2*) mutation (Pareyson *et al.*, 2000), *dynactin 1* (*DCTN1*) mutation (Puls *et al.*, 2003, 2005), distal HMN type VII linked to chromosome 2q14 (McEntagart *et al.*, 2001) and a few patients with *CMT1A* duplication (Thomas *et al.*, 1997). In our screening cohort, 5 out of the 14 patients who initially presented with vocal fold paralysis were shown to have a *TRPV4* mutation (36%). This indicates that *TRPV4* is likely to be a major gene for hereditary neuropathies with vocal fold paralysis. Why the recurrent laryngeal nerve innervating the vocal folds is so vulnerable for the effects of *TRPV4* mutations remains unclear at this point. However, the predominant involvement of the left vocal fold could result from length dependency since the left recurrent laryngeal nerve descends around the aortic arch before turning to go up to the larynx, making it much longer than its counterpart on the right.

The additional feature of bladder urgency and urinary incontinence was observed in members of family F1 and in patient CMT-858.09. This finding is more commonly seen in the context of spinal cord disorders and autonomic neuropathies, but is highly unusual in the context of hereditary (non-autonomic) neuropathies. Therefore, more patient observations and systematic bladder function studies are needed in order to attribute this feature to the *TRPV4* disease spectrum. No other autonomic disturbances were noted in any of the patients.

In the context of the broad phenotypic variability of *TRPV4* mutations, special interest goes to patient PN-1394.1 carrying the *de novo* Val620Ile mutation, which was previously shown to cause autosomal dominant brachyolmia (Rock *et al.*, 2008). None of the *TRPV4*-associated skeletal dysplasia syndromes are known to feature peripheral neuropathy or other neurological involvement. The skeletal abnormalities in patient PN-1394.1 were obvious but too limited to make a formal diagnosis of a skeletal dysplasia. Particularly the abnormalities of the spine could also be secondary to the longstanding neuropathy as was previously suggested (Auer-Grumbach *et al.*, 2010). None of the more common neuropathy genes were mutated in this individual, making the incidental co-occurrence of a mutation in another neuropathy-associated gene unlikely. Taking the overall variability of *TRPV4*-associated disorders into account, patient PN-1394.1 may represent an overlap syndrome between skeletal dysplasia and neuropathy. Skeletal abnormalities such as congenital hip dysplasia, scoliosis, small hands and limb shortening have been observed in the scapuloperoneal SMA family with the Arg316Cys mutation (DeLong and Siddique, 1992; Deng *et al.*, 2010). The authors already suggested a *TRPV4* disease spectrum encompassing both peripheral neuropathy and skeletal dysplasia

(Deng *et al.*, 2010). Our patient PN-1394.1 might be a first confirmation of this intriguing hypothesis.

All known neuropathy-causing *TRPV4* mutations (except Val620Ile) are surface mutations not expected to affect protein folding. They cluster on the highly positively charged convex surface of the ARD and target arginine residues that are strictly conserved throughout 27 available *TRPV4* orthologues, suggesting their importance in *TRPV4* function (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landoure *et al.*, 2010) (Table 1 and Fig. 3B). These mutations are located in three consecutive finger loops of the protein, Arg232Cys in finger 2, Arg269Cys/Arg269His in finger 3 and Arg315Trp/Arg316Cys in finger 4 (Fig. 3A–C). In contrast, the bone dysplasia mutations (Ile331Phe and Asp333Gly) (Krakow *et al.*, 2009) are situated on the opposing concave side (Fig. 3A). This concave surface of ankyrin repeats is commonly used in protein–protein interactions, whereas few ankyrin-repeat-containing proteins have ligands known to bind to the convex surface (Gaudet, 2008).

TRPV4 mutations causing skeletal dysplasias probably act through a gain of function mechanism (Rock *et al.*, 2008; Krakow *et al.*, 2009). For the neuropathy-causing mutations however, discrepancies exist in the current reports. In one study, three *TRPV4* mutations (Arg269His, Arg315Trp and Arg316Cys) influenced proper localization of the ion channel to the plasma membrane (Auer-Grumbach *et al.*, 2010) with the formation of cytoplasmic aggregates. Mutant *TRPV4* was shown to cause loss of normal channel function (Auer-Grumbach *et al.*, 2010). The results from two other studies differed substantially (Deng *et al.*, 2010; Landoure *et al.*, 2010); both showed increased intracellular calcium levels caused by abnormal activity of the *TRPV4* ion channel in the presence of either the Arg269His, Arg269Cys or Arg316Cys mutation. The use of various cell systems in the above-mentioned studies may underlie the differences in results (Nilius and Owsianik, 2010). Nevertheless, it remains uncertain whether changes in *TRPV4* channel function alone are causing the peripheral neuropathy. Further studies are definitely needed to shed light on the disease mechanism.

Since the majority of neuropathy-causing *TRPV4* mutations cluster on one face of the ARD, disturbances in protein–protein interactions specific to nervous system form an interesting pathophysiological hypothesis; however, this has not been proven so far (Landoure *et al.*, 2010). The only mutation outside of the ARD in this study is the *de novo* Val620Ile mutation in patient PN-1394.1, which may argue against this hypothesis. Alternatively, it may only imply that this mutation works through a different disease mechanism. Although the neuropathy-associated mutations seem to cluster in well-defined residues of the ARD, the skeletal dysplasia mutations are more spread out over the protein including two in the ARD, Asp333Gly and Ile331Phe (Krakow *et al.*, 2009). This means that an absolute distinction between the two groups of phenotypes cannot be made based on the position of the mutation in the primary structure of the protein alone. The skeletal abnormalities in the original scapuloperoneal SMA family (Deng *et al.*, 2010) with the Arg316Cys mutation and patient PN-1394.1 with the Val620Ile mutation seem to strengthen this point further.

A parallel could be made with dominant mutations in *dynamitin 2* (*DNM2*) causing an intermediate form of HMSN and centronuclear myopathy. Overall, mutations causing one of the two phenotypes cluster in different domains of the protein but the distinction is not absolute (Claeys *et al.*, 2009). Likewise, the majority of *lamin A/C* (*LMNA*) mutations causing one of the many laminopathies cluster together in certain domains of the protein; however, some mutations have been reported to cause distinct phenotypes in different patients (Rankin and Ellard, 2006). Given the substantial phenotypic variability of *TRPV4*-related disorders, additional modulating factors, both genetic and environmental, may be at play (Auer-Grumbach *et al.*, 2010; Deng *et al.*, 2010; Landouere *et al.*, 2010).

In addition to the substantial phenotypic heterogeneity of *TRPV4*-associated neuropathy, genetic heterogeneity in pedigrees with similar phenotypes has also been reported (Landouere *et al.*, 2010). In a second family with congenital distal SMA, linkage to the 12q23–q24 locus was excluded (van der Vleuten *et al.*, 1998). A distal HMN type VII with vocal fold paresis was suggested to be allelic with HMSN2C, however the disease-causing locus was mapped to chromosome 2q14 (McEntagart *et al.*, 2001).

In this study we enlarge the genetic spectrum of *TRPV4*-related disorders and report considerable phenotypic variability of hereditary neuropathies caused by *TRPV4* mutations. Nearly all patients in our series have one or more features that are rather unusual for the most commonly encountered hereditary neuropathies. Conversely, screening of 62 HMSN2 phenotypes revealed only one family with a *TRPV4* mutation (CMT-1100). Therefore we propose to direct diagnostic screening of *TRPV4* towards patients presenting with either pure motor, or motor and sensory neuropathies, with clear axonal electrophysiology and at least one of the following additional clinical features: vocal fold paralysis, proximal weakness in the upper limbs, pronounced scoliosis or contractures. While performing mutation analysis of *TRPV4*, the extensive phenotypic variability and reduced penetrance should be taken into account, especially in the context of genetic counselling.

In order to shed more light on the underlying pathomechanism through which *TRPV4* mutations work, further research into the extent of the associated disease spectrum is warranted. This could include the study of the contribution of *TRPV4* in patients with distal HMN without additional features or patients with milder skeletal abnormalities in the absence of neuropathy (e.g. idiopathic scoliosis). Also the degree of phenotypic overlap between skeletal dysplasia and peripheral neuropathy could be explored further by means of systematic nerve conduction exams in *TRPV4* skeletal dysplasia patients, a study that has not yet been performed to our knowledge.

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Supplementary material

Supplementary material is available at *Brain* online.

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